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Heparin enhances the furin cleavage of HIV-1 gp160 peptides

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Abstract **Infectious HIV-1 requires gp160 cleavage by furin at the REKR^{511}↓ motif (*site1*) into the gp120/gp41 complex, whereas the KAKR^{503} (*site2*) sequence remains uncleaved. We synthesized 41mer and 51mer peptides, comprising *site1* and *site2*, to study their conformation and *in vitro* furin processing. We found that, while the previously reported 19mer and 13mer analogues represent excellent *in vitro* furin substrates, the present extended sequences require heparin for optimal processing. Our data support the hypothesis of a direct binding of heparin with *site1* and *site2*, allowing selective exposure/accessibility of the REKR sequence, which is only then optimally cleaved by furin.**

Keywords: HIV-1; Furin; Heparin; Glycosaminoglycan; gp160; Processing

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the etiological agent for the acquired immunodeficiency syndrome (AIDS). The envelope glycoprotein gp160 is processed by host proteases into the gp120/gp41 heterodimer. This allows the virus to infect target cells following the cell surface binding of the trimeric complex gp120/gp41 to the human CD4 receptor [1], and subsequently to the CXCR4/CCR5 co-receptors [2,3]. This interaction induces conformational change, which leads to the dissociation of gp120 from gp41, allowing the N-terminal gp41 fusion peptide to be inserted into the host cell membrane [4]. Though the structures of a monomeric gp120 core in complex with the CD4 receptor/Fab 17b [5] and that of a post-fusion form of gp41 were solved [6], little is known about the entire *Env* conformation. In fact, individually gp120 does not mimic its precursor conformation, as two monoclonal antibodies directed against the V3 loop recognized gp160, but not gp120 [7].

The *Env* precursor is cleaved by the proprotein convertase (PC) furin or furin like-proteases at the REKR^{511}↓ *site1* [8], resulting in the fusogenic gp120/gp41 complex. Since the R511T mutation results in uncleaved gp160 and non-infectious virus, cleavage is essential for viral entry [9]. *In vitro* investigations on peptides encompassing the gp120/gp41 junction confirmed that cleavage occurs at Arg^{511}↓ [10]. Interestingly, upstream of the physiological processing site, a second *site2* potential furin motif (KAKR^{503}) is inefficiently cleaved. The exact role of *site2* is unknown, though mutations of gp160 KAKR^{503} sequence result in an unprocessed precursor [11]. Conformational differences between *site1* and *site2* may explain the preference of furin for *site1* [12].

Even though short gp160 peptides are efficiently cleaved *in vitro* by furin at Arg^{511}, the full length gp160 was shown not to be efficiently cleaved *ex vivo*. This suggests that gp160 structure [11,13], post-translational modifications [14], as well as cellular and/or extracellular factors, may also influence the efficacy and selectivity of the furin mediated cleavage [15]. Viruses, such as Sindbis [16] and coronavirus [17], bind to target cells *via* cell-surface glycosaminoglycans (GAGs). Proteoglycans were also shown to facilitate HIV-1 binding to and/or entry into cells lacking the CD4 receptor [18]. Moreover, enzymatic removal of cell surface heparan sulfate chains drastically impairs HIV-1 infection of CD4+ cells [19]. This effect likely implicates gp160 and/or gp120 interaction with GAGs [20–22]. Many authors identified the binding site for heparin or its derivatives within the gp120 V3 loop [21]. However, an increasing body of evidence points to a possible gp160–GAGs interaction [22].

To investigate the influence of the sequence surrounding the REKR^{511} motif on gp160 processing efficiency, we synthesized various peptides derived from the gp120/gp41 junction (Table 1). Moreover, as a measure of the modulating role of GAGs on the furin processing, we evaluated the effect of heparin by both circular dichroism (CD) and HPLC. We found that the gp120/gp41 junction itself binds heparin, thus enhancing its furin processing.

2. Materials and methods

2.1. Peptide synthesis

The synthesis of the 14mer and 19mer was reported [10]. The 18mer, 41mer, and 51mer were synthesized on a semi-automatic synthesizer (Applied Biosystems, Mod. 431A) using a Rink amide MBHA resin

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Abbreviations: HIV-1, human immunodeficiency virus type 1; GAGs, glycosaminoglycans; CD, circular dichroism; AIDS, acquired immunodeficiency syndrome; RP-HPLC, reverse phase high performance liquid chromatography; PC, proprotein convertase; BTMD, before trans membrane domain; MS, mass spectrometry; AMC, 7-amino-4-methyl-coumarin; MCA, 7-amido-4-methylcoumarin; TFE, trifluoroethanol; SDS, sodium dodecyl sulfate; Tris–HCl, Tris-(hydroxymethyl) aminomethane–HCl; DMSO, dimethyl sulfoxide; HF, hydrofluoric acid; cmk, chloromethylketone; PBS, phosphate buffer saline

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Table 1
Peptide sequences

Name	Sequence
51mer	^{484}YKYKVVKIEPLGVAPT**KAKR**RVVQ**REKR**↓AVGIGALFLGFLGAAGSTMGAAS^{534}
41mer	^{494}LGVAPT**KAKR**RVVQ**REKR** AVGIGALFLGFLGAAGSTMGAAS^{534}
18mer	^{494}LGVAPT**KAKR**RVVQ**REKR**^{511}
19mer	^{498}PT**KAKR**RVVQ**REKR** AVGIG^{516}
13mer	^{504}RVVQ**REKR** AVGIG^{516}

(NovaBiochem, La Jolla, 0.48 mmol/g, 0.25 mmol), Boc chemistry and HBTU/HOBt activation. Detachment from the solid support and removal of the side chain protecting groups were achieved treating with HF:anisole:DMSO:2-mercaptopyridine/10:1:1:1 (1 h, 0 °C). Crude products were purified by reverse phase high performance liquid chromatography (RP-HPLC) on a Delta Pak HR C_{18} column (Waters, 6 μm, 60Å, 7.8 × 300 mm). Homogeneity grade was evaluated by RP-HPLC on a Vydac C_{18} column (Waters, 5 μm, 300 Å, 4.6 × 250 mm). Molecular mass was checked by electrospray-time of flight (TOF) mass spectrometry (MS; Mariner 5120 API-TOF).

2.2. Circular dichroism

CD spectra were recorded on a Jasco CD spectropolarimeter Model J-710 with a cylindrical fused quartz cell (path length 0.1 cm). The spectra are reported in units of mean ellipticity (peptide molecular weight/number of amide bonds), $[\Theta]_R$ (deg cm^2 dmol^{-1}) or ellipticity, $[\Theta]$ (deg cm^2). The measurements were performed in water, 10 mM phosphate buffer pH 7, 14 mM sodium dodecyl sulfate (SDS) in 10 mM phosphate buffer pH 7, trifluoroethanol (TFE) 98%, 20 μM heparin (Sigma, low molecular weight) in 0.15 M NaCl + 25 mM Tris-(hydroxymethyl) aminomethane–HCl (Tris–HCl) buffer pH 7, and in 0.15 M NaCl + 25 mM Tris–HCl buffer pH 7. Peptide concentrations, determined by amino acid analysis or UV absorption, varied from 18 to 43 μM. The spectra were corrected for the solvents, salts and heparin minor contributions.

2.3. Recombinant hfurin

The media of BSC40 cells infected with either wild type vaccinia virus (VV:WT, control) or a soluble form of hfurin (VV:hfurin-BTMD) [8] were collected 18 h post-infection and concentrated (Centriprep YM-30). Activity was measured with the fluorogenic substrate Pyr-RTKR-MCA.

2.4. Enzymatic assays

Assays were performed in 100 μL at 37 °C on 100 μM peptide in 2 mM $CaCl_2$, 25 mM Tris–HCl buffer pH 7.0, 1 mM β-mercaptoethanol, 2 μL furin (~2 relative fluorescence units (RFU); where 1 RFU is defined as 1 pmol 7-amino-4-methyl-coumarin (AMC) released/min/μl enzyme acting on 100 μM of the fluorogenic substrate Pyr-RTKR-MCA) or as control 2 μL of media from VV:WT culture supernatant. When specified, incubation media also contained 2.5 μM, 20 μM or 25 μM heparin. Heparin alone shows no enzymatic activity (*not shown*). At various time points, 20 μL samples were analyzed by RP-HPLC on a Varian C_{18} column (5 μm, 100 Å, 4.5 × 250 mm) and digestion products identified by mass spectrometry (MS). Percent cleavage was calculated from precursor areas.

2.5. Inhibition assays

Reactions, performed in 100 μL (25 mM Tris–HCl, 1 mM β-mercaptoethanol and 2 mM $CaCl_2$, pH 7.0) at 37 °C, contained 50 μM Pyr-RTKR-MCA or 100 μM 19mer as substrate, 2 μL of furin and different concentrations (1–100 μM) of the 18mer as inhibitor or 5 μM dec-RVKR-cmk [23]. Enzymatic activity with MCA-conjugated peptidyl substrate was monitored (360 nm excitation, 460 nm emission) with a Spectra MAX GEMINI EM microplate spectrofluorometer (Molecular Devices), in the presence or absence of either 20 μM or 100 μM heparin. Inhibition assays with the 19mer were monitored by RP-HPLC. The IC_{50} were calculated using GraFit Version 4.09 software.

3. Results

3.1. Peptides design

Four peptides, 51mer, 41mer, 19mer and 13mer, spanning the gp160 cleavage sequence, were synthesized (Table 1). The 13mer containing *site1*, and the 19mer, which includes *site1* and *site2*, were chosen as Ref. [10]. The extended 41mer and 51mer were synthesized to investigate the influence of the regions surrounding the physiological cleavage site on furin processing. It was reported that a cell-permeable 22mer sequence KIEPLGVAPTKAKRRVVQREKR^{511}, which does not contain P′ residues, interferes with gp160 processing [24]. Thus, to test for a possible *in vitro* inhibitory function we also synthesized a 18mer peptide (LGVAPTKAKRRVVQ-REKR^{511}), mimicking the furin-processing product of the 41mer (Table 1).

3.2. Circular dichroism

The spectra in phosphate buffer pH 7.0 and water showed a diagnostic band with a minimum at 198 nm, suggesting that the 51mer, 41mer and 19mer are unstructured (Fig. 1A–C). In the presence of SDS, a red shift of the negative band was observed with a minimum at 201 nm for the 19mer (Fig. 1A). Interestingly, based on the 220 nm band intensity, the same micellar solution induced the 41mer and 51mer to assume a 20% and 21% α-helix conformation respectively (Fig. 1B,C). This indicates a likely SDS interaction that could be due to the insertion of the hydrophobic C-terminus into micelles and/or could result from the electrostatic binding of the positively charged *site1* and/or *site2* to the negatively charged micellar surface.

TFE induced order in the structure of the 19mer, 41mer, and 51mer (positive band at 190 nm, two negatives at 206 and 220 nm). The α-helix content was 30% in 98% TFE/H_2O for the 19mer, 59% for 41mer and 62% for 51mer (Fig. 1A–C).

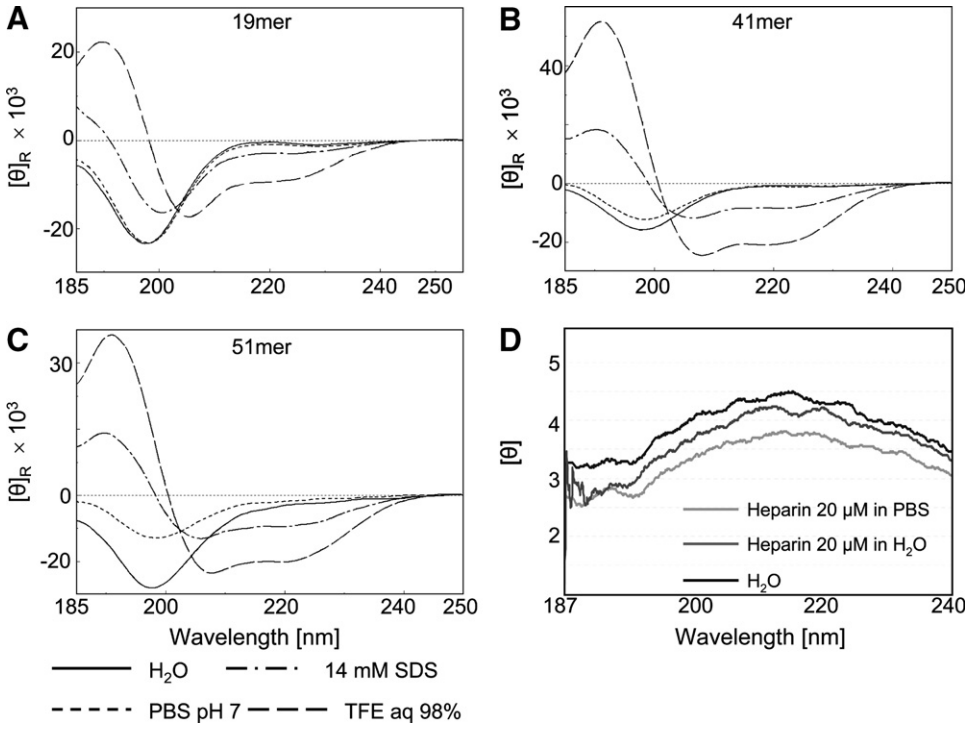

Fig. 1. CD spectra of: (A) 19mer, (B) 41mer, and (C) 51mer in (——) water, (- - - - -) Phosphate buffer pH 7, (-·-·-·-) 14 mM SDS, and (– – –) 98%TFE/H_2O; (D) water (black), 20 μM heparin (dark gray) in H_2O and phosphate buffer pH 7.2 (light gray).

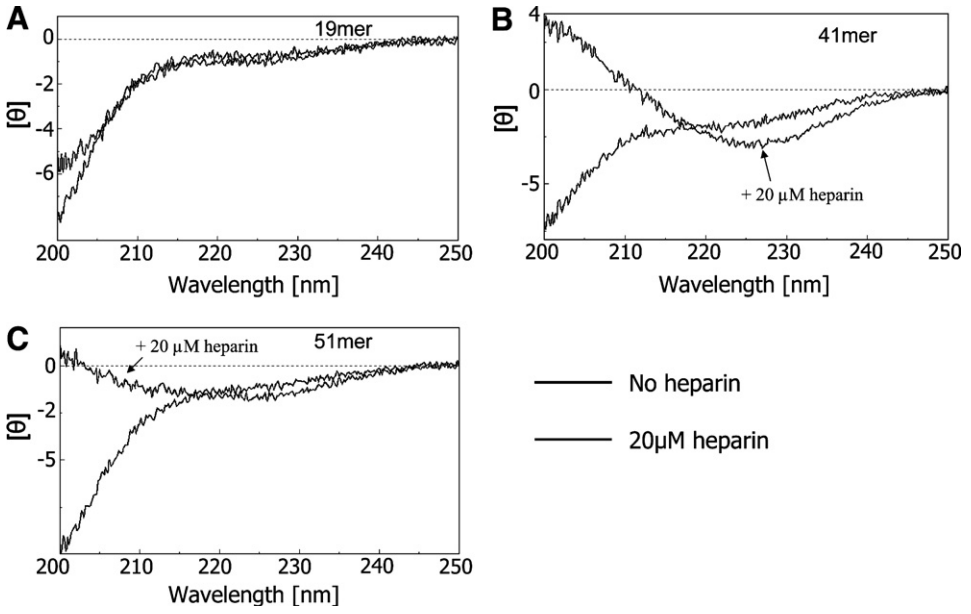

Fig. 2. CD spectra of: (A) 19mer, (B) 41mer, and (C) 51mer in (black) 0.15 M NaCl and 25 mM Tris–HCl buffer pH 7, and (gray, arrow) 20 μM heparin in 0.15 M NaCl and 25 mM Tris–HCl buffer pH 7.

Since CD profiles in negatively charged SDS micellar solutions showed a transition of conformers towards a more structured population, and gp160 cleavage site is positively charged, further conformational investigations were performed. The CD profile of 20 μM heparin is similar to that of water (Fig. 1D) and that of the 19mer does change in the presence of heparin (Fig. 2A). In contrast, the CD spectra of the 41mer and the 51mer were significantly modified in the presence *versus* absence of heparin (Fig. 2B,C). Similar results were obtained with higher heparin concentrations (100 μM).

3.3. Enzymatic assays

The 13mer and 19mer peptides were digested equally well by furin at *site1* (Tables 2 and 3), showing complete processing at 5 h (Fig. 3A,B). In contrast, the 41mer and 51mer peptides were either barely or unprocessed, respectively, even after

Table 2
Percent cleavage of the 13mer, 19mer, 41mer, and 51mer upon 2 h and overnight incubation in presence or absence of heparin

Time incubation [h]	13mer		19mer		41mer		51mer	
	Heparin							
	–	+	–	+	–	+	–	+
	% Cleavage							
0	0		0		0		0	
2	53	54	59	52	0	32	0	8
6	100	100	100	100	0		0	
Overnight					Traces	40	0	60

Table 3
Fragments sequences derived from cleavage at *site1* or *site2* and their corresponding theoretical and experimental masses

Precursor	Fragments sequence	Theoretical mass (Da)	Experimental mass (Da)
13mer	RVVQREKR	1069.65	1070.26
	AVGIG	415.24	415.27
18mer	LGVAPTKAKR	1069.65	Undetectable
	RVVQREKR	1039.65	Undetectable
	PTKAKRRVVQREKR	1751.08	1751.16
19mer	AVGIG	415.24	415.27
	PTKAKR	699.44	Undetectable
	RVVQREKRAVGIG	1466.88	Undetectable
	LGVAPTKAKRRVVQREKR	2091.29	2091.30
41mer	AVGIGALFLGFLGAAGSTMGAAS	2039.06	Undetectable
	LGVAPTKAKR	1039.65	Undetectable
	RVVQREKRAVGIGALFLGFLGAAGSTMGAAS	3090.69	Undetectable
	YKYKVVKIEPLGVAPTKAKRRVVQREKR	3339.01	Undetectable
51mer	AVGIGALFLGFLGAAGSTMGAAS	2039.06	Undetectable
	YKYKVVKIEPLGVAPTKAKR	2287.38	Undetectable
	RVVQREKRAVGIGALFLGFLGAAGSTMGAAS	3090.69	Undetectable

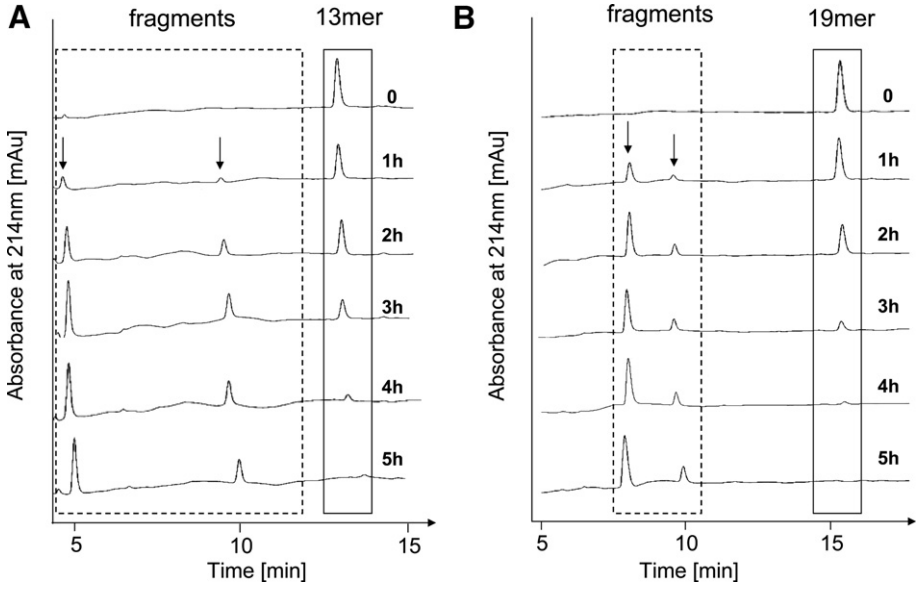

Fig. 3. RP-HPLC profiles of: (A) 13mer digestion and (B) 19mer digestion obtained on a Varian C_{18} column with UV detector (214 nm). Arrows indicate the fragments.

24 h digestion at pH 7 (Fig. 4A,B; Tables 1–3). Since *in vitro* gp160 cleavage was reported to be optimal at pHs 6–7 [25], further assays were performed on the 41mer and 51mer at acidic conditions (pH 6.3, 6.7), again revealing no differences with respect to the results obtained at pH 7. Furthermore, similar data were observed in presence of low levels of denaturants (0.05% TX-100 or SDS) (*not shown*).

We first hypothesized that product inhibition could explain these results. We thus tested the *in vitro* ability of the 18mer peptide, representing the furin-derived product of the 41mer (Table 1), to inhibit the processing of either the fluorogenic Pyr-RTKR-MCA or the 19mer peptides. While the 18mer peptide effectively reduced the release of free AMC with an estimated IC_{50} of 1.6 μM (Fig. 5A), it could only partially

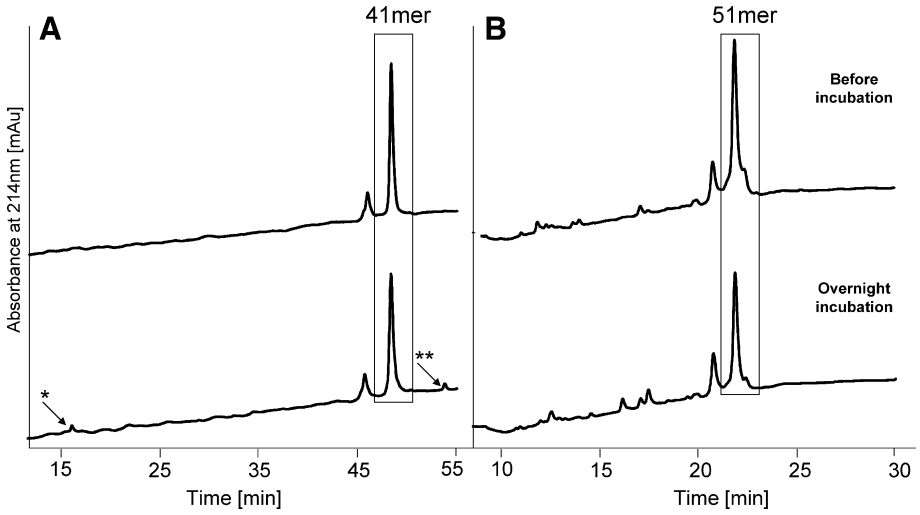

Fig. 4. RP-HPLC profiles of the digestion of the (A) 41mer and (B) 51mer obtained on a Varian C_{18} column with UV detector (214 nm). 20 μL of the enzymatic assay solution was taken (top) immediately after the addition of the substrate and upon overnight incubation (bottom). Arrows indicate the fragments, (*) being LGVAPTKAKRRVVQREKR for the 41mer and (**) being AVGIGALFLGFLGAAGSTMGAAS.

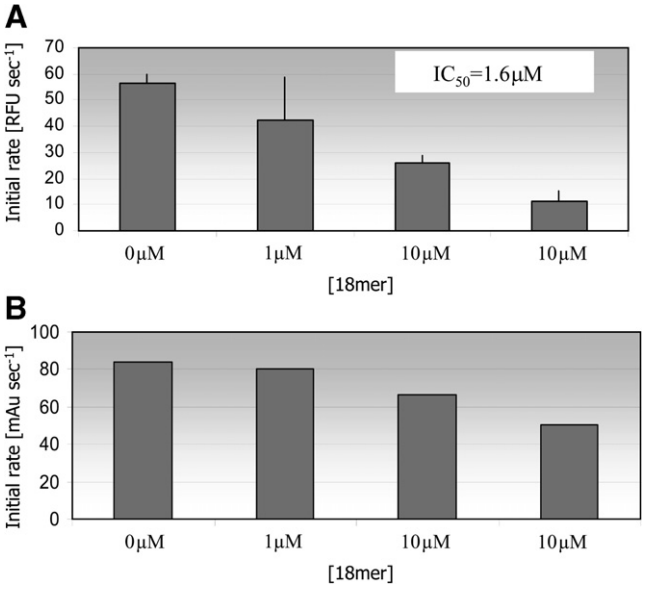

Fig. 5. Initial rate versus concentration of the 18mer to assess its effect on the furin cleavage of: (A) Pyr-RTKR-MCA, and (B) 19mer.

inhibit the 19mer processing with an $IC_{50} > 100$ μM (Fig. 5B). We conclude that product inhibition cannot explain the inability of furin to process the 41mer and 51mer peptides.

Because CD investigations showed a likely binding between heparin and the 41mer or 51mer (Fig. 2B,C), all four gp160-derived analogues were digested overnight in the absence or presence of 2.5 μM heparin. Under these conditions, the 13mer and 19mer were digested at *site1* with similar rates independent of the presence of heparin. In contrast, while no significant processing occurred in the absence of heparin, ~40% and ~60% processing at the REKR↓ site of the 41mer (into an 18mer product with identical retention time on RP-HPLC to the synthetic version) and 51mer peptides, respectively, were observed in the presence of 2.5 μM heparin (Fig. 6). As control, we confirmed that the 41mer peptide is not cleaved by the recombinant VV:WT-infected culture supernatant (Fig. 6, upper center panel). Furthermore, cleavage was inhibited by adding a well known PC-inhibitor, dec-RVKR-cmk (Fig. 6, upper right panel) [23]. At 25 μM heparin we obtained a more extensive processing, but also noticed precipitation of the peptides (*not shown*). Finally, in a separate 6 h furin incubation experiment, the processing of the 41mer and 51mer peptides also showed a similar enhancement effect of heparin (*not shown*). In conclusion, these data indicate a likely heparin-peptide interaction that may better expose *site1*, and hence allow more effective furin cleavage.

4. Discussion

Five peptides (Table 1) spanning the gp120/gp41 junction were investigated to better define the gp160 glycoprotein cleavage. The 19mer and its shorter analogue 13mer were processed by furin at *site1* ($REKR^{511}$), while *site2* ($KAKR^{503}$), which is included only in the 19mer, was uncleaved (Fig. 3). The lack of processing at *site2* may be rationalized on the basis of structural motifs. In fact, the 19mer NMR molecular model in TFE revealed that *site2* is embedded in a helical segment, whereas *site1* is in a exposed loop at the C-terminus of the peptide [12]. In contrast, the 41mer and 51mer, spanning extensive sequence of the gp160 cleavage region, were shown to represent very poor furin substrates. This suggests that the generated fragments could either act as inhibitors or that the more extended regions surrounding the physiological cleavage site prevents effective processing.

Since the possibility of product inhibition by the 18mer was excluded, we turned our attention towards structural restrictions and/or the need of other factors to rationalize the non-cleavability of the 41mer and 51mer peptides. CD analysis on the 19mer, 41mer and 51mer in aqueous solution revealed that the three analogues are unstructured, and yet only the 19mer is digested by furin. Thus, some structural constraints must exist in the 41mer and 51mer, at least around *site1*. The same argument may explain the inability of furin to cleave

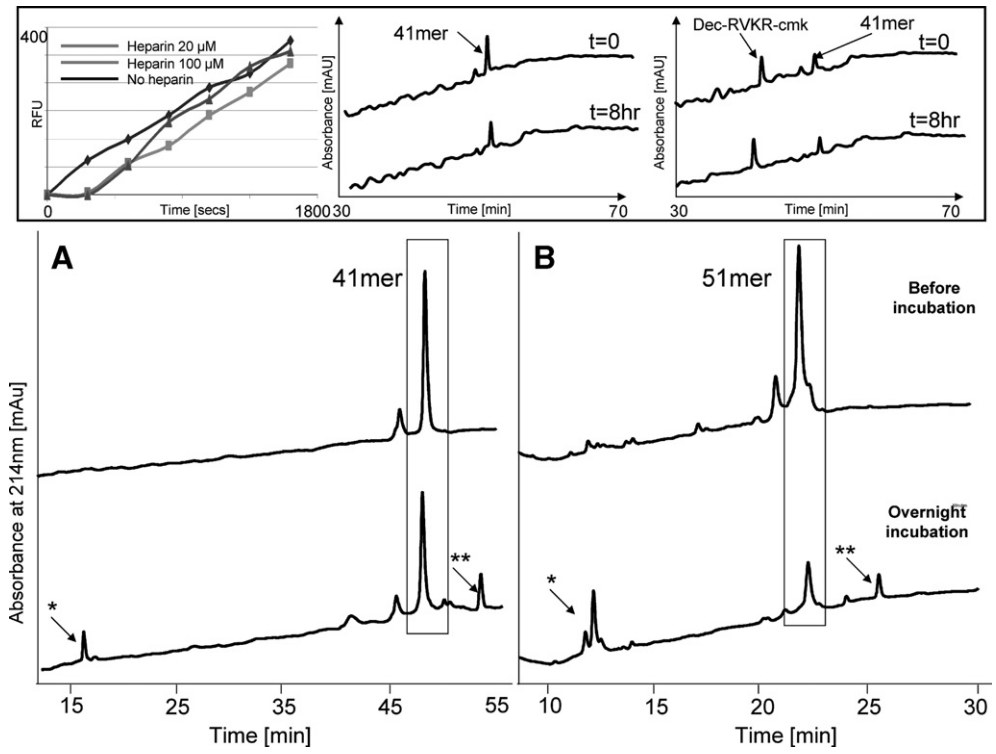

Fig. 6. Effect of heparin on the processing of the 41mer and 51mer. Upper panel controls: (left) RFU released versus time upon Pyr-RTKR-MCA cleavage by furin in (black) the absence, or presence of 20 μM (dark gray) or 100 μM (light gray) heparin; (upper central panel): incubation of the 41mer peptide with recombinant vaccinia WT-infected culture supernatant (control); (upper right panel) effect of decanoyl-RVKR-cmk on the processing of the 41mer by furin. processing. Lower panels: 20 μL of the enzymatic assay solution containing 2.5 μM heparin was taken (top) immediately after the addition of the substrate and (bottom) after overnight incubation. Arrows indicate the fragments, (*) being LGVAPTKAKRRVVQREKR for the 41mer peptide or YKYKVVKIEPLGVAPTKAKRRVVQREKR for the 51mer peptide and (**) being AVGIGALFLGFLGAAGSTMGAAS.

at *site2* in any substrates used. In an attempt to increase the 41mer and 51mer processing, we added some detergents to enhance the peptides backbone flexibility without affecting enzyme activity. However, neither TX-100 nor SDS had any effect.

Therefore, we suspected that cellular/extracellular factors may influence the cleavability of gp160 by furin. Indeed, surface proteins containing heparin-binding motifs processed by furin were reported [16,26]. In particular, Sindbis virus attachment to target cells was enhanced in the presence of heparan sulfate (HS) *via* the furin recognition motif of the unprocessed envelope glycoprotein PE2 [16]. Similarly, peptides derived from the cleavage site of the human respiratory syncytial virus (RSV) fusion glycoprotein bind heparin and cellular GAGs [26]. Since the gp120/gp41 does not form stable trimers, while unprocessed gp160 does, it was hypothesized that gp160 oligomer attachment to the plasma membrane heparin sulfate occurs *via* its furin cleavage site [27]. Indeed, the **K**A**KR**^{503}**R**VVQ**R**E**KR**^{511} sequence exhibits a basic region, which contains two potential inverted *consensus* HS-binding domains. Thus, it was shown that the affinity of gp160 for heparin is about 3-times higher than that observed for gp120, implying that gp41 and/or hidden motifs in the mature gp120 may be involved in heparin binding [22].

CD spectra of the 41mer and 51mer suggest these peptides could interact with heparin (Fig. 2) and undergo structural reorganization. In fact, in presence of heparin, the profiles change with respect to those of the peptides alone. The negative band at 198 nm, diagnostic for aperiodic structures, is replaced by a positive one. In contrast, it is noteworthy that the shorter 19mer does not change its CD profile in the presence of heparin. Since the difference between the 41mer and the 19mer lies in 22 hydrophobic residues and the interaction between heparin and polypeptides is supposed to be electrostatic, these additional residues may support a favorable peptide conformation that optimally orients the positively charged side chains towards the negatively charged sulfate moieties. Our results agree with a probable glycosaminoglycans-gp160 interaction, as proposed [22,27], and suggest that the residues spanning the gp120/gp41 junction may contribute in gp160-GAGs binding.

Moreover, given that heparin induces a change in the 41mer and 51mer conformation, which could play a key role in the enzyme-substrate recognition, we analyzed how it may influence their furin processing. Surprisingly, while up to 100 μM heparin did not influence furin activity on Pyr-RTKR-MCA processing (Fig. 6, left upper panel), the 41mer and 51mer peptides were digested at *site1* (Fig. 6). Therefore, we hypothesize that heparin induces conformational change, optimally exposing the furin-cleavage REKR^{511} site. This is the first time that heparin is shown to enhance the *in vitro* cleavage of precursors by furin.

In conclusion, this study has shown that, in the absence of heparin, the 41mer and 51mer gp160 derived peptides represent very poor furin substrates *in vitro*, in contrast to the shorter analogues (13mer and 19mer) that are efficiently processed.

Heparin was shown to strongly interact with the 41mer and 51mer peptides, inducing conformational changes, thereby exposing *site1* for cleavage. Since the 41mer and 51mer peptides may not faithfully mimic the conformation around the cleavage site within the complete gp160 precursor, more analyses are required to assess if heparin is essential *in vivo* during gp160 maturation and how GAGs modulate HIV-1 activity.

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